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Large Deletion of the X-Linked Lymphoproliferative Disease Gene Detected by Fluorescence In Situ Hybridization

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The X-linked lymphoproliferative disease (XLP) is an inherited immunodeficiency characterized by an abnormal responses to infection with Epstein–Barr virus (EBV), resulting in fatal infectious mononucleosis, hypogammaglobulinemia, virus-associated hemophagocytic syndrome, and malignant lymphoma. Mutations in the gene coding for a T cell-specific SLAM-associated protein (SAP) have been recently identified in XLP patients. We report on a 1-year-old boy representing fulminant hemophagocytic syndrome. He developed high fever, lymphadenopathy, hepatosplenomegaly with liver dysfunction, and pancytopenia with marrow hemophagocytosis. EBV DNA was abnormally increased in the blood. Polymerase chain reaction failed to amplify SAP mRNA and genomic DNA products from the patient's peripheral blood. A large deletion of the SAP gene was confirmed by fluorescence in situ hybridization (FISH). FISH analysis also disclosed that the patient's mother was a carrier. We conclude that FISH can be useful in the diagnosis of XLP with large deletions of the SAP gene and its carrier state. Am. J. Hematol. 64: 128–132, 2000.

Key words: X-linked lymphoproliferative disease; SAP gene; gene deletion; fluorescence in situ hybridization

INTRODUCTION

X-linked lymphoproliferative disease (XLP), also known as Duncan's disease, is an inherited immunodeficiency disorder characterized by an extremely high sensitivity to primary infection with Epstein–Barr virus (EBV). As a result, patients with XLP develop a complex syndrome of severe or fatal infectious mononucleosis, aplastic anemia, hypogammaglobulinemia, malignant B-cell lymphoma, and sometimes virus-associated hemophagocytic syndrome [1–3].

Genetic linkage studies have localized the XLP locus to the long arm of the human X chromosome in Xq24-Xq25 [4,5]. Restriction fragment length polymorphism analysis using several DNA markers closely linked to the XLP locus has been used for female carrier detection and identification of boys at risk for XLP before EBV infection [6–8].

Recently, three independent groups have provided evidence that XLP results from mutations or deletions of SH2 domain-encoding gene [9,10], or SLAM (signaling lymphocyte-activation molecule)-associated protein (SAP) gene [11]. In this report, we describe a patient who

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presented with a fulminant case of EBV-related hemophagocytic syndrome. Polymerase chain reaction (PCR) could not amplify products of the coding region for the SAP gene from the patient's peripheral blood. The presence of a large deletion in the SAP gene was confirmed by fluorescence in situ hybridization (FISH) of peripheral blood mononuclear cells (MNCs). FISH analysis also demonstrated that the patient's mother was a carrier.

CASE REPORT

A 1-year-3-month-old boy was admitted to the local hospital because of fever, diarrhea and the occurrence of a generalized seizure. The high-grade fever continued, and swelling of the tonsils and cervical lymphadenitis ensued. The diagnosis of severe infectious mononucleosis was made, and the patient was referred to our hospital. Although he had no siblings, his mother's younger brother died of severe viral infection at 1 year of age. Physical examination at admission disclosed high fever, respiratory failure, and a maculopapular rash on his trunks. Other findings included marked tonsillar swelling and several cervical lymphadenopathy; liver and spleen were palpable at 10 cm and 11 cm under the costal margin, respectively. On laboratory examination, white blood cell count was 21.4×10^9/L with 71% atypical lymphocytes, hemoglobin level was 7.9 g/dL, and platelet count was 53×10^9/L. Blood gas revealed hypoxia with 32.3 mmHg of pO_2. Prothrombin time and partial thromboplastin time were prolonged to 14.5 sec (*N:* 9.0–12.0 sec) and 55.2 sec (*N:* 26.0–36.0 sec), respectively. Hypoproteinemia and liver dysfunction were also observed as follows: total protein, 5.6 g/dL (*N:* 6.4–8.3 g/dL); aspartate aminotransferase, 2,198 IU/L (*N:* 13–33 IU/L); alanine aminotransferase, 453 IU/L (*N:* 8–42 IU/L); lactic dehydrogenase, 6,032 IU/L (*N:* 261–483 IU/L). Serum ferritin level was elevated to 19,795 ng/mL (*N:* 5–130 ng/mL). Immunoglobulin (Ig) levels were 1,570 mg/dL in IgG (*N:* 800–1,800 mg/dL), 543 mg/dL in IgA (*N:* 100–300 mg/dL), and 1,200 mg/dL in IgM (*N:* 60–200 mg/dL). The number of activated T lymphocytes (HLA-DR+CD3+ cells) increased to 44.5% (*N:* <10%). The IgG and IgM antibody titers to EBV capsid antigen (VCA) were 1:20 and <1:10, respectively, whereas antibodies to EBV early antigen (EA) and nuclear antigen (EBNA) were negative. Peripheral blood MNCs was found to contain 33,873 copies of EBV DNA/μg [12]. Bone marrow included 42% of atypical mature or immature lymphoid cells with azulophilic granules and 10% of histiocytes with hemophagocytic activity. These atypical cells were negative for both peroxidase and esterase stainings, and most of them showed positive reaction for HLA-DR and anti-CD3 monoclonal antibodies by surface marker analysis. The diagnosis of acute lymphoid leukemia was therefore excluded in this patient. Chromosomal analysis showed a normal karyotype with 46,XY. An increase of histiocytic cells (50/μL) was also observed in the cerebrospinal fluid. Magnetic resonance imaging of the brain showed no abnormal findings. On the basis of a diagnosis of EBV-related hemophagocytic syndrome, treatment with etoposide (150 mg/m^2, weekly) and dexamethasone (10 mg/m^2/day for 2 weeks) was initiated. Fever, respiratory failure and cervical lymphadenopathy gradually ameliorated, whereas hepatosplenomegaly and hyperferritinemia continued. One week after initiation of chemotherapy, high fever recurred and cyclosporine A was added to the treatment. Despite these therapies, the patient died of respiratory failure and severe bacterial infection associated with pancytopenia 3 weeks after the admission.

MATERIALS AND METHODS

RNA and DNA were extracted from peripheral blood MNCs by using guanidine isothiocyanate–phenol (Trizol, GibcoBRL, Rockville, MD). cDNA was prepared by reverse transcription at 42°C for 50 min in a 20 μL mixture containing 1 μg of RNA and 0.5 ng of random hexamer, using the SuperScript preamplification systems (GibcoBRL). Reverse transcription (RT)-PCR was performed by using primers for amplification of SAP mRNA: 5′-GCCTGGCTGGCGTAGCAGGGGCATCTCCC-3′ and 5′-ATGTACAAAAGTCCATTTCAGCTTTGAC-3′ [11]. Four exons of the SAP gene were amplified by PCR using the following primers: exon 1, 5′-GCCCTACGTAGT GGGTCCACATACCAACAG-3′ and 5′-GCAGGAGGCCCAGGGAATGAAATCCCCAGC-3′; exon 2, 5′-GGAAACTGTGGTTGGGCAGATACAATATGG-3′ and 5′-GGCTAAACAGGACTGGGACCAAAATTCTC-3′; exon 3, 5′-GCTCCTCTTGCAGGGAAATTCAGCCAACC-3′ and 5′-GCTACCTCTCATTTGACTTGCTGGCTACATC-3′; exon 4, 5′-GACAGGGACCTAGGCTCAGGCATAAACTGAC-3′ and 5′-ATGTACAAAAGTCCATTTCAGCTTTGAC-3′ [11]. The expected size of each PCR product was 629 bp for SAP mRNA, 839 bp for exon 1, 443 bp for exon 2, 484 bp for exon 3, and 515 bp for exon 4, respectively. PCR was performed with cDNA corresponding to 100 ng RNA or DNA in a total volume of 50 μL, containing 1 × PCR buffer, 200 μM NTPs, 0.2 μM each of the 5′ and 3′ primers, and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT). The amplification profile involved an initial denaturation step of 10 min at 95°C, and 40 cycles of denaturation at 95°C for 30 sec, primer annealing at 53°C for 30 sec, and extension at 72°C for 1 min. The PCR products were resolved by electrophoresis in 1% agarose gels, stained with ethidium bromide, and visualized under UV light. PCR for β-actin

and GAPDH was used as a control for RNA and DNA analysis, respectively.

Peripheral blood MNCs stimulated in culture with phytohemagglutinin (PHA) for 72 hr were treated with hypotonic solution (4:1 0.075 M KCl and 1% sodium citrate) and fixed with Carnoy's solution (3:1 methanol and acetic acid) for metaphase preparation. The SAP gene specific genomic probe used in FISH analysis was prepared by PCR using exon 2 sense primer and exon 4 antisense primer as described above. The probe, labeled with digoxigenin-11-dUTP using PCR labeling [13], was hybridized to metaphase samples as previously described [14]. The biotin-labeled X chromosome centromeric probe (DXZ1, Oncor, Gaithersburg, MD) was used to recognize the X chromosome. The SAP specific probe was detected with anti-digoxigenin rhodamine (Boehringer Mannheim, Germany) providing a red signal, while DXZ1 was detected with avidin fluorescein (Vector Laboratories, Burlingame, CA) providing a green signal. Metaphases were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and the images of the hybridization were captured under fluorescence microscopy (Olympus Optical Co., Tokyo, Japan). At least 20 metaphases were observed for each sample.

RESULTS

We asked whether a mutation in the XLP gene might be responsible for the refractory EBV-related hemophagocytic syndrome he had developed, because one of his relatives died of severe viral infection in infancy. Genetic analysis of the SAP gene in the patient was performed two weeks after the admission. The results of PCR analysis for the SAP gene are shown in Figure 1. Compared with healthy controls, no RT-PCR product for SAP mRNA was amplified from the patient's RNA (Fig. 1A). DNA-PCR analysis for SAP exons 1, 2, 3, and 4 also revealed no amplification products, suggesting that this patient carried a large DNA deletion that included the SAP gene (Fig. 1B).

FISH analysis using a combination of a SAP specific probe and the X-chromosome specific DXZ1 probe was performed. In the four samples obtained from normal controls (two males and two females), one signal from the SAP gene probe was observed on metaphases from male samples, and two signals were detected from female samples. Signals from metaphases of all normal samples observed had adequate fluorescent signal intensity, indicating that the assay was suitable for detection of the SAP gene. The results of FISH analysis in the patient and his parents are shown in Figure 2. No SAP specific signal was observed in metaphases from the patient. One signal for the SAP gene was detected in metaphases from the father. Notably, only one signal for the SAP gene was detected in metaphases from the mother. These results were consistent in every metaphase chromosome from

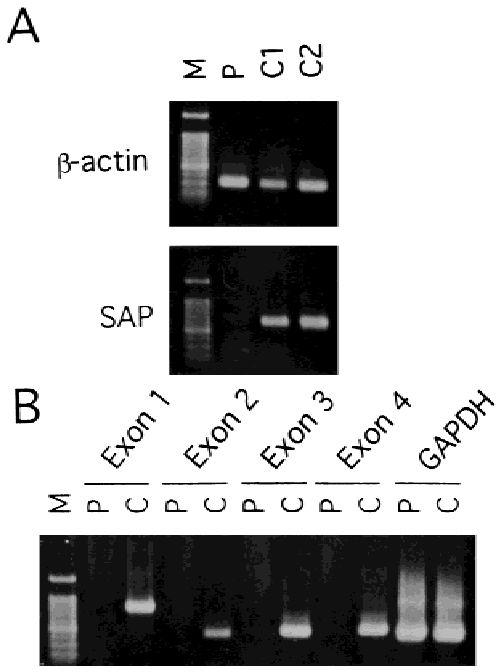

Fig. 1. PCR analysis of the SAP gene in the patient and healthy controls. (A) RT-PCR analysis of SAP mRNA expression in peripheral blood mononuclear cells (629 bp). (B) DNA-PCR for exon 1 (839 bp), exon 2 (443 bp), exon 3 (484 bp), and exon 4 (515 bp). PCR for β-actin and GAPDH was used as an internal control for mRNA and DNA, respectively. P, patient; C, healthy control; M, 100 bp DNA markers.

the patient, the father, and the mother. These results indicate that this patient carried a large deletion of the SAP gene and that the patient's mother was an obligate carrier of the deletion.

DISCUSSION

Identification of the molecules that are defective in XLP has the potential to provide an understanding of the regulatory mechanisms that underlie critical immune responses to EBV infection. Patients with XLP display an array of abnormal and exuberant responses to EBV infection, including uncontrolled expansions of $CD8^+$ T cells, EBV-infected B cells and macrophages in various tissues of the body [3]. Even prior to infection with EBV, these patients often develop immunodeficiencies such as hyper-IgA or IgM, hypo-IgG1 or IgG3, or failure to undergo IgM to IgG switch after secondary challenge with ϕX174 [15]. This instability to mount an IgG response is often reflected in the failure of XLP patients to mount an IgG anti-EBNA after EBV infection [3]. In our patient, hyper-IgM was noted at admission. Despite the high levels of EBV DNA in the blood, the titers of VCA-IgG and IgM antibodies were only 1:20 and <1:10, whereas antibodies to EA and EBNA were undetectable. In Japan, an elevated titer of VCA-IgM antibody was observed in only 40% of cases of less than 3 years of age with EBV-induced infectious mononucleosis [16]. Our patient can

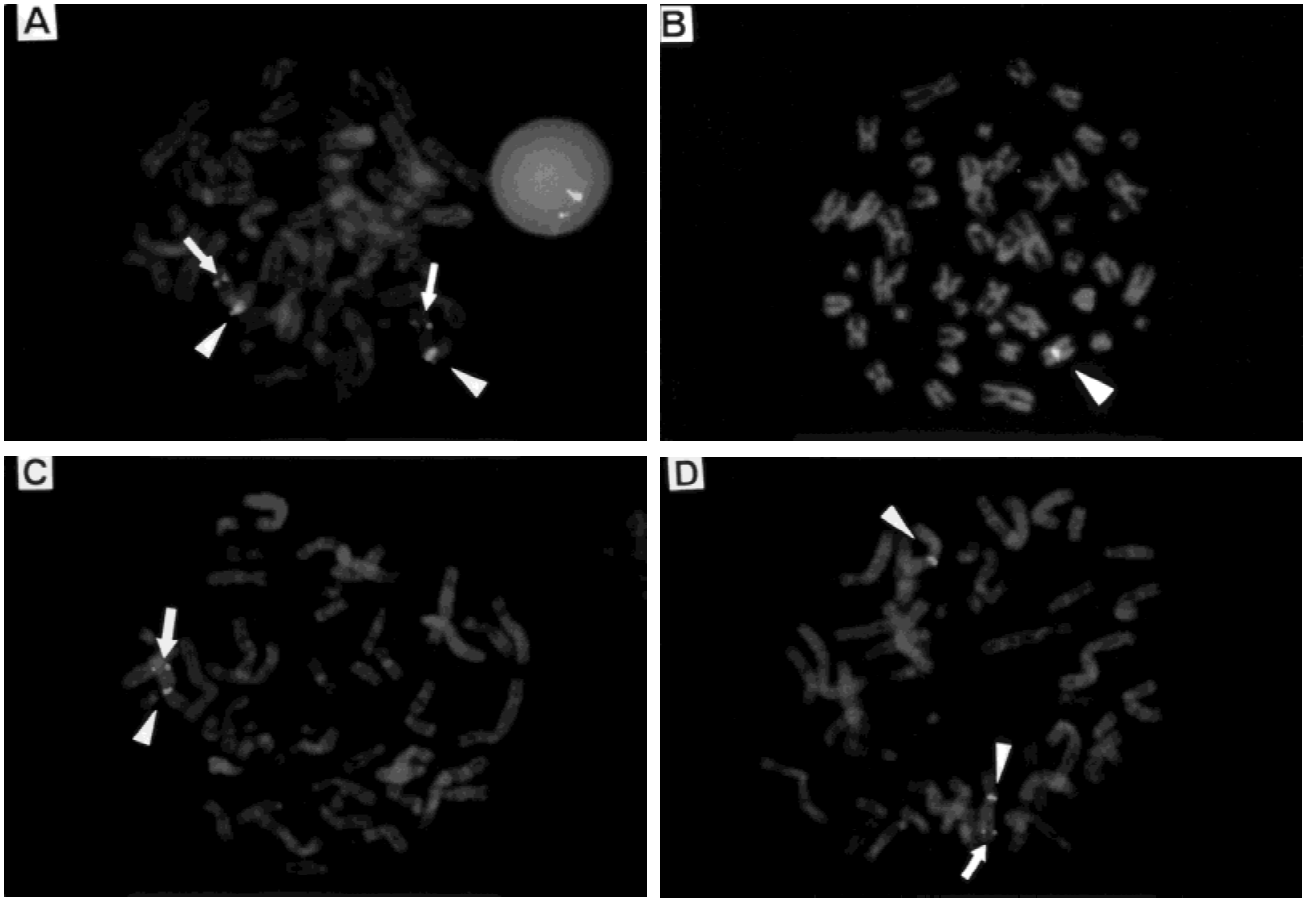

Fig. 2. FISH analysis with a SAP specific probe and the X-chromosome specific DXZ1 probe. The triangles identify the chromosome X localization of the centromeric probe (DXZ1), which gave two signals in the healthy female (A) and in the mother of the patient (D); a single signal in the patient (B) and his father (C). Large arrows identify the localization of the SAP specific probe. Two SAP related signals were detected from the healthy female, one in each X chromosome (A), and one signal was detected in the single X chromosome from the father (C). The patient (B) displayed no signal from the SAP probe, and the mother displayed only one signal (D).

be therefore diagnosed as having a primary EBV infection, although the impaired serological responses to EBV infection were observed.

Previous genetic linkage studies localized the XLP gene to Xq24-q25, distal to DXS42 and DXS37 [17]. Analysis with Xq25 specific probes identified deletions in Xq25 in patients with XLP and disease carriers, the smallest of which was estimated to be approximately 3 Mb [18]. Recently, Coffey et al. [9] defined the critical region for XLP to be between DXS6791 and DXS100 from patients with the smallest of these deletions, and identified the SH2 domain-containing gene (SH2D1A) as the candidate for XLP. Nine different mutations of SH2D1A were described in XLP patients: point mutations in 8 patients, and deletion of 159 bp in one patient [9]. Independently, Sayos et al. [11] identified proteins that interact with SLAM (for signaling the lymphocyte-activation molecule) on the surface of B and T cells. Because the gene coding for this SLAM-associated protein (SAP) localized in Xq25, these investigators examined whether the SAP gene could be involved in XLP and found that the SAP gene contained a point mutation in one patient and a large deletion in two related patients [11]. In addition, Nichols et al. [10] described the presence of small deletions and intragenic mutations that specifically disrupt the SH2 domain-encoding gene, named as DSHP in 6 of 10 unrelated XLP patients. Since sequence analysis showed that these three genes are identical, these findings demonstrate that XLP results from mutations of the SAP/SH2D1A/DSHP gene, and suggest that this gene plays a critical role in the regulation of lymphocyte activation and proliferation.

XLP has been thought to be absent or extremely rare in Japan. Since the SAP gene has been linked to the pathogenesis of XLP, mutations (missense, nonsense, or small deletion) of this gene were recently identified in several Japanese patients with and without a family history (Sumazaki and Kanegane et al., manuscript in preparation). In the present study, we describe the occurrence of a large genomic deletion of SAP gene in a patient who developed a fatal case of EBV-related hemophagocytic syndrome. FISH analysis confirmed the presence of a

genomic deletion of the SAP gene. By this method, the patient's mother displayed a deletion of the SAP gene in one X chromosome, indicating that she was a carrier of the disease.

SAP deletions have previously been detected in other XLP patients. Genomic deletions that span the entire DSHP gene and a large deletion of the entire XLP locus were characterized in XLP patients [10]. Another report also showed that the SAP gene was deleted in two of the three XLP patients whose DNA was analyzed by PCR [11]. No SAP mRNA was detected by Northern blot analysis in these patients [11]. Our study shows that FISH analysis can be a useful tool to diagnose XLP patients and obligate carriers with large deletions of SAP gene. Absence of detectable mutations or deletion in some XLP patients could be explained on the basis of mutations of the SAP gene in intronic sequences or promoter region [9,10]. The involvement of other genes in some XLP patients cannot be excluded.

Thus, FISH analysis can be used successfully for diagnosis of XLP female carriers and affected males before exposure to EBV. We do not know whether patients with large deletions or point mutation of SAP gene display different clinical and immunological syndromes. Nor do we fully understand what functions the SAP gene product plays in immune function. Use of a simple test such as FISH will likely extend our ability to recognize XLP patients and carriers and may permit the implementation of preventive therapies prior to EBV infection [19,20].

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